

## Changes in a Murine Leukemia Virus (MLV) Receptor Encoded by an Alphavirus Vector during Passage in Cells Expressing the MLV Envelope

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We constructed alphavirus vectors encoding the ecotropic murine leukemia virus (MLV) receptor mCAT1. Cells electroporated with these vector RNAs expressed mCAT1 protein and fused with cells that expressed a fusogenic form of the MLV envelope on the cell surface. Electroporated cells also released submicron particles that were infectious in envelope-expressing cells. Infection resulted in giant syncytia that could be enumerated by plaque assay. Cell-free supernatants could be serially passaged and contained up to  $10^4$  infectious units/ml. To determine whether repeated passage would select for functional variants of the receptor, we analyzed mCAT1 amplified by RT-PCR after 12 serial passages. Several amino acid substitutions were identified that encoded functional receptor variants. In independent experiments, variants containing an isoleucine or a leucine instead of a phenylalanine at position 224 in the third extracellular domain of the receptor arose spontaneously and outgrew the parental vector, indicating that mutations at this site are highly selected in this system. This region of the receptor has previously been implicated in the envelope–receptor interaction. This alphavirus vector system provides a novel method for generating and selecting functional variants of viral receptors © 2000 Academic Press

**Key Words:** alphavirus; primitive virus; murine leukemia virus; envelope; ecotropic; receptor.

### INTRODUCTION

Expression vectors have been made from alphaviruses by substituting genes of interest for alphavirus structural genes (Xiong *et al.*, 1989; Liljestrom and Garoff, 1991). The alphavirus genome consists of a single-stranded RNA ~12 kb long, the first two thirds of which encodes an enzyme complex that replicates viral RNA via a negative-strand intermediate (Strauss and Strauss, 1994). Transcription of the virus negative strand results in full-length genomic RNA as well as a subgenomic, positive-strand RNA corresponding to the 3' end of the genome that encodes capsid and envelope. Substitution of other genes for capsid-envelope yields vectors that, when introduced as RNA into susceptible cells, go through one round of RNA amplification and produce large amounts of the substituted gene product. These vectors do not spread between cells due to the absence of virus structural proteins.

In 1994, Rolls *et al.* (1994) observed that an alphavirus vector encoding vesicular stomatitis virus envelope glycoprotein (VSV-G) could spread in tissue culture, despite the absence of alphavirus structural proteins, due to fusogenic activity of VSV-G protein. Sonicates of vector-containing cells were capable of initiating new rounds of infection. Supernatants of infected cells also contained

infectious particles, with titers of  $\sim 10^4$  infectious units/ml. The infectivity was resistant to RNase, sensitive to detergent, could be filtered ( $<0.45 \mu\text{m}$ ), banded in sucrose, and neutralized by anti-VSV-G antiserum, suggesting that it consisted of membranous vesicles with VSV-G protein on the outside and vector RNA on the inside. Because the infectious particles lacked capsid, they were dubbed "primitive viruses." Spontaneous release of infectious vesicles could have been related to cytopathicity of VSV-G protein or the alphavirus replicon, which shuts off host cell synthesis and leads to cell death in 2–3 days. We observed that alphavirus vectors encoding a fusogenic form of MLV envelope also generated infectious vesicles that could be passaged like viruses in cells expressing the cognate receptor (Lebedeva *et al.*, 1997).

Given the apparent structural simplicity of these primitive viruses, we wondered whether the roles of envelope and receptor could be switched (i.e., if vectors encoding virus receptor could generate vesicles capable of infecting cells expressing virus envelope on their surface). In a rhabdovirus vector system, vectors encoding CD4 plus HIV coreceptor were reported to make particles that were infectious specifically for cells bearing HIV envelope (Schnell *et al.*, 1997; Mebatsion *et al.*, 1997). MLV vectors encoding retroviral receptors instead of MLV envelope were also recently shown to be infectious for cells expressing the cognate envelope (Baillet and Bates, 1998). The receptor for the ecotropic class of MLV

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is a cell surface protein that normally functions as a cationic amino acid transporter, mCAT1 (Albritton *et al.*, 1989; Kim *et al.*, 1991; Wang *et al.*, 1991). We constructed alphavirus vectors encoding mCAT1 and investigated their ability to spread infection in cells expressing MLV envelope. We found that such vectors could be serially passaged as cell-free supernatants and that serial passage was associated with selection of functional variants of the receptor. This represents a novel system for generating and selecting functional variants in virus receptors.

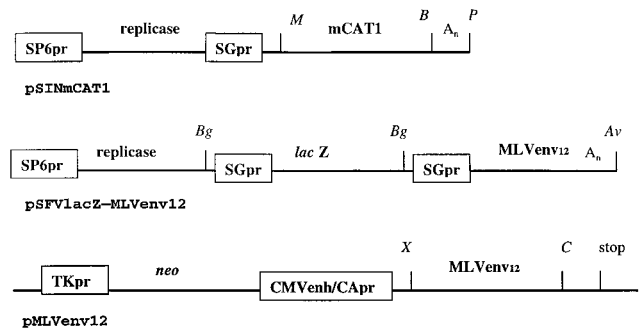
## RESULTS

### Vectors encoding an MLV receptor are infectious in cells expressing a fusogenic form of MLV envelope

MLV envelope is synthesized as a nonfusogenic precursor that is activated by cleavage of 16 amino acids from the cytoplasmic tail of its transmembrane (TM) segment (Ragheb and Anderson, 1994; Rein *et al.*, 1994). This cleavage, which is normally effected by the viral protease late in the process of virus budding, produces a 12-kDa form of TM ("p12e") (Schultz and Rein, 1985). The predominant form of envelope on the surface of infected cells is the immature, nonfusogenic form. We previously showed that wild-type MLV envelope expressed from an alphavirus vector was not fusogenic because it did not undergo the activating cleavage of its cytoplasmic tail, whereas MLV envelope truncated by virtue of a stop codon at the site of the cytoplasmic tail cleavage site was fusogenic (Lebedeva *et al.*, 1997).

BHK cells electroporated with a Sindbis vector encoding the MLV receptor mCAT1 (Fig. 1, line 1) formed syncytia when cocultivated with BHK cells electroporated with vectors encoding the truncated MLV envelope. They did not form syncytia when cocultivated with cells expressing the full-length, immature envelope on the cell surface such as NIH3T3 cells chronically infected with MLV and BHK cells electroporated with vectors encoding the full-length MLV envelope. Similar results were obtained with Semliki Forest virus vectors encoding mCAT1.

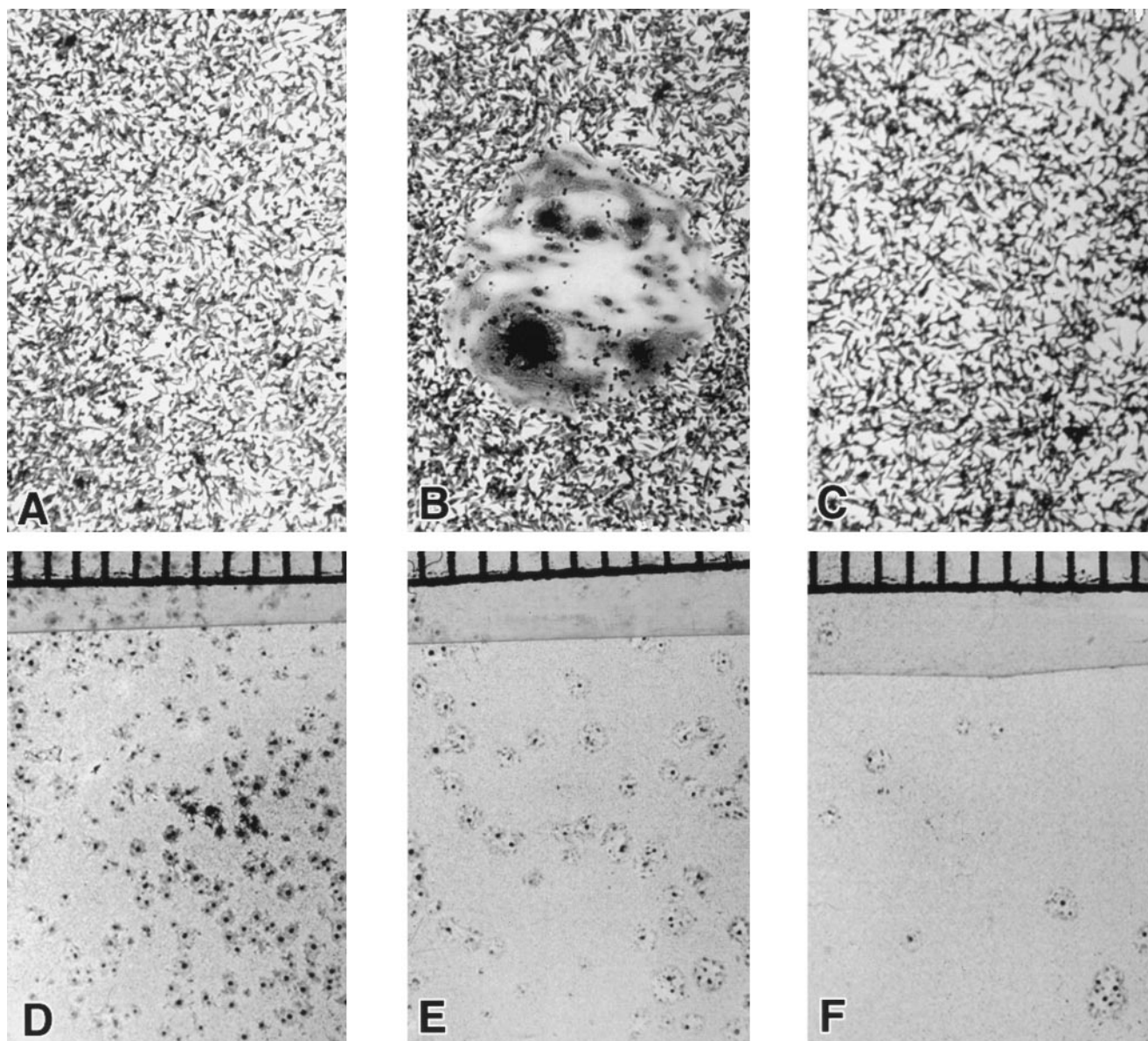
To determine whether cells electroporated with alphavirus vectors encoding mCAT1 produced infectious vesicles, we first constructed target cells that stably expressed the fusogenic form of MLV envelope on their surface. We transfected BHK cells with a stable expression plasmid pMLVenv12 (Fig. 1, line 3) and selected G418-resistant colonies. Colony cells were tested for expression of MLV envelope by flow cytometry using an antiserum to MLV envelope. Several MLV envelope positive clones were established; one used in subsequent experiments is designated BHK-MLVenv12. BHK-MLVenv12 cells formed syncytia when cocultivated with cells expressing mCAT1 (XC cells, NIH3T3 cells, and BHK cells electroporated with alphavirus vectors encoding mCAT1). They did not form syncytia with mCAT1-negative



**FIG. 1.** Schematic diagram of plasmid constructs. Nonrelevant portions of plasmids are omitted. The first two lines show the structure of plasmids from which alphavirus vector RNA was synthesized *in vitro*. SP6pr denotes the SP6 RNA polymerase promoter used for RNA synthesis; replicase, the alphavirus nonstructural genes that recognize and replicate virus-related RNA *in vivo*; SGpr, the alphavirus subgenomic promoter, which determines the 5' end of subgenomic viral RNAs; mCAT1, the ecotropic MLV receptor; A<sub>n</sub>, 3' poly(A)<sup>+</sup> tract encoded in the vector just upstream of the vector linearization site; lacZ,  $\beta$ -galactosidase; and MLVenv12, fusogenic form of MLV envelope with a stop codon at the normal p12e cleavage site. The last line shows the structure of the DNA expression plasmid used to make indicator BHK cells stably expressing the fusogenic form of MLVenv. TKpr denotes thymidine kinase promoter; *neo*, G418 resistance gene; CMVenh/CATpr, CMV immediate-early enhancer followed by a chicken actin promoter; and stop, stop codon inserted at the end of MLV env p12e. Restriction sites: M, *Mlu*I; B, *Bsp*120I; P, *Pac*I; X, *Xba*I; Bg, *Bgl*II; Av, *Ava*I; and C, *Cla*I.

cells, including BHK cells and MLV-NIH3T3 cells, in which the mCAT1 receptor is absent from the cell surface due to chronic infection with MLV.

BHK-MLVenv12 cells were then used as an indicator cell line to assay for infectious particles capable of directing the synthesis of mCAT1. To determine whether BHK cells electroporated with the Sindbis mCAT1 vector could make such particles, we disrupted these cells by Dounce homogenization, sonication, or vigorous shaking with glass beads; filtered the material through 0.45- $\mu$ m filters; and infected BHK-MLVenv12 cells with the filtrate. Syncytia appeared after ~8 h, the time required for alphavirus vectors to replicate and express passenger genes (Garoff and Li, 1998). Syncytia gradually increased in size until they were nearly 1 mm in diameter at 48–72 h (Fig. 2B), after which they began to slough. This time course suggests that newly synthesized mCAT1 protein was responsible for syncytia rather than mCAT1 protein in the inoculum. The three disruption methods produced approximately equal titers:  $\sim 10^4$  to  $10^5$  syncytia-forming units/ml. The infectious inocula did not produce syncytia on plain BHK cells (Fig. 2C), NIH3T3 cells, or MLV-NIH3T3 cells, which express the full-length MLV envelope. As a further control, we made NIH3T3 cells stably expressing the truncated, fusogenic MLV envelope. Because plain NIH3T3 cells die when transfected with pMLVenv12, presumably because they fuse with neighboring cells expressing mCAT1, we used MLV-infected NIH3T3 cells in which the mCAT1 receptor is down-



**FIG. 2.** Syncytia and plaque assay. Low-power photomicrographs of cells stained with carbol Fuchsin-methylene blue (see Materials and Methods). (A-C) The fields of view are  $\sim 1 \times 2$  mm. (A) Uninfected BHK-MLVenv12 cells. (B) BHK-MLVenv12 cells infected with filtered supernatant from BHK cells electroporated with pSINmCAT1 RNA. (C) BHK cells infected with the same supernatant as in B. (D-F) Lower-power view of BHK-MLVenv12 cells infected with 1 ml, 100  $\mu$ l, or 10  $\mu$ l, respectively, of infectious material derived from pSINmCAT1 as in B. In D-F, the bar markings at the top are in mm.

regulated. MLV-NIH3T3 cells were transfected with pMLVenv12 and selected with G418, yielding a clone designated MLV-NIH3T3-MLVenv12. These cells formed syncytia when infected with sonicates from BHK cells electroporated with SINmCAT1 vectors. Thus syncytia formation by the SINmCAT1 sonicates were specific for cells expressing the fusogenic envelope.

#### Growth kinetics of CAT1 infectious vectors on MLV envelope-expressing cells

BHK-MLVenv12 cells were infected with 0.5 ml of filtered sonicate from BHK cells electroporated with pSIN-

mCAT1. Four hours after infection, the inoculum was replaced with fresh medium. Supernatants and cells from replicate dishes were collected at various times thereafter. The supernatants were filtered (0.45  $\mu$ m), and the cells were sonicated in 1 ml of medium and then filtered (0.45  $\mu$ m). The filtrates were assayed for syncytia-inducing units on BHK-MLVenv12 cells. Figures 2D-2F provide an example of the plaque assay.

In the first experiment summarized in Table 1, an inoculum containing 650 syncytia-inducing units was used to infect BHK-MLVenv12 cells at a multiplicity of infection of  $\sim 10^{-3}$ . Lysates of these infected cells were

TABLE 1

## Growth Kinetics of Infectious Vesicles

		Syncytia-inducing units in indicated volume		
		0.1 ml	0.01 ml	0.001 ml
Cell lysate	4	0	0	0
	8	0	0	0
	16	TMTC	20	4
	24	TMTC	70	13
	32	TMTC	100	36
	48	85	13	5
		0.5 ml	0.05 ml	0.005 ml
Supernatant	8	1	1	0
	16	28	8	1
	24	76	9	3
	32	TMTC	55	15
	48	TMTC	TMTC	200

*Note.* Experiment I: BHKenv12 cells were infected with 1 ml of filtered cell lysate containing 659 syncytia-inducing units of SINmCat1 vector. Four hours later, the inoculum was replaced with 3 ml of DMEM. Supernatants and cells were collected at the indicated times from replicate dishes; cells were lysed in 3 ml of DMEM, and lysates and supernatants were filtered (0.45 μm) and assayed for syncytia-inducing units on fresh BHK-MLVenv12 cells.

Hours postinfection	4	8	12	20	30	48
Cell homogenate titer (/ml)	10	1000	7000	28,000	5000	5000
Supernatant titer (/ml)	80	16	10	100	200	200

*Note.* Experiment I: BHKenv12 cells were infected with 1 ml of filtered cell lysate containing 659 syncytia-inducing units of SINmCAT1 vector. Four hours later, the inoculum was replaced with 3 ml of DMEM. Supernatants and cells were collected at the indicated times from replicate dishes; cells were lysed in 3 ml of DMEM, and lysates and supernatants were filtered (0.45  $\mu$ m) and assayed for syncytia-inducing units on fresh BHK-MLVenv12 cells.

*Note.* Experiment II: BHK-MLVenv12 cells were infected with 5000 syncytia-inducing units and assayed as for experiment I.

first detected to contain syncytia-inducing units 16 h after infection, and the number of syncytia-inducing units increased from 16 to 32 h after infection and then declined. The decline was associated with an extensive cytopathic effect in the producer cultures. The peak titer in the cell lysate was  $4 \times 10^4$  syncytia-inducing units/ml, which indicates that the culture produced more than 100 infectious units for each input infectious unit. Syncytia-inducing units also were detected in the supernatant, increasing from 16 to 48 h after infection, with a peak of  $4 \times 10^4$  infectious units/ml.

In the second experiment summarized in Table 1, the inoculum contained 5000 syncytia-inducing units,  $\sim 10$  times the previous dose. The titer in the cell lysate peaked earlier, at 20 h, at  $2.8 \times 10^4$  infectious units/ml. Spontaneously released infectious material was lower, 200 infectious units/ml, but as in the first experiment, it persisted for 48 h. The amount of spontaneously released infectivity has varied between experiments much more than the amount of cell-associated infectivity and may be sensitively related to the input titer, target cell density, and degree of cytopathicity.

## Selection of CAT1 variants during serial passage

Alphavirus vectors mutate rapidly in tissue culture due to low fidelity of the viral replicase plus selection for variants that replicate and/or spread faster (Strauss and Strauss, 1994). In vectors that contain passenger genes not required for spread or replication, the additional genes are often rapidly lost (Pugachev *et al.*, 1996; Rolls *et al.*, 1996). Therefore, we theorized that repeated passage of the Sindbis-mCAT1 vector on BHK-MLVenv12 cells might result in the loss of portions of the mCAT1 gene that are not required for spread in this system or reveal other mutations in the receptor that either were neutral or facilitated spread.

As a preliminary test of this idea, we made a Semliki vector encoding  $\beta$ -galactosidase followed by a duplicated subgenomic promoter driving the fusogenic MLV env (Lebedeva *et al.*, 1997) (Fig. 1, line 2). RNA from this vector induced  $\beta$ -galactosidase-positive syncytia in NIH3T3 cells. Sequential passage of filtered supernatants from these cells produced syncytia in NIH3T3 cells, but the proportion of syncytia that expressed  $\beta$ -galactosidase decreased from 54 of 54 (100%) on passage 1 to 88 of 112 (79%) on passage 2 to 64 of 104 (72%) on passage 3 to 26 of 68 (38%) on passage 4 and 2 of 202 (1%) on passage 5. Thus a gene for which there was no selection ( $\beta$ -galactosidase) was rapidly lost on passage of infectious vector.

To determine what happened to the mCAT1 gene on passage, after 12 serial passages of filtered supernatants, we amplified the  $\sim 2$ -kb mCAT1 insert by RT-PCR and cloned the amplified product in the empty Sindbis vector, pSinRep5. Sequencing the insert in three clones revealed one in-frame deletion of three amino acids, two silent base changes, nine base changes resulting in amino acid substitutions in the coding sequence, as well as three substitutions and one single base insertion in the 3' noncoding region (Table 2). All three clones were functional in terms of ability to induce syncytia and infectious particles in electroporated BHK-MLVenv12 cells. One of the changes, a T-to-A substitution at position 670 corresponding to a phenylalanine-to-isoleucine substitution at residue 224 in the third putative extracellular domain of CAT1, was common to all three clones. This region of mCAT1 has previously been implicated in the envelope-receptor interaction (Albritton *et al.*, 1993; Kavanaugh *et al.*, 1994; Kim and Cunningham, 1993; Yoshimoto *et al.*, 1993; Eiden *et al.*, 1993, 1994).

To determine whether these changes arose during serial passage, rather than as artifacts of PCR, we made use of the fact that some of the changes lead to the gain or loss of a restriction site. This allowed us to analyze bulk RT-PCR products from infected cells at various levels of passage for the presence of the variant sequences. The phenylalanine-to-isoleucine change at amino acid 224 corresponds to loss of a *Tsp*509I site at

TABLE 2

Nucleotide and Predicted Amino Acid Changes in mCAT1 after 12 Serial Passages.

Clone	Nucleotide position	Amino acid position
1 <sup>a</sup>	670 T → A	224 Phe → Ile
1	1380 C → T	460 silent
1	1562 C → T	521 Ala → Val
1	1781 T → C	594 Phe → Ser
1	2090 insert C	3' noncoding
1	2153 C → T	3' noncoding
2	114 A → T	38 silent
2	299 A → G	100 Tyr → Cys
2 <sup>a</sup>	670 T → A	224 Phe → Ile
2	790 T → C	264 Cys → Arg
2	851 T → C	284 Val → Ala
2	1875 A → G	3' noncoding
3 <sup>a</sup>	670 T → A	224 Phe → Ile
3	936 C → G	312 Ile → Met
3	1283 A → G	428 Glu → Gly
3	1743–1751 deletion	582–584 (Phe-Ala-Val) <sub>del</sub>
3	1811 C → T	604 Ala → Val
3	1894 A → G	3' noncoding

Note. Position 1 = first base of mCAT1 coding sequence.

<sup>a</sup> Common to all clones.

position 667. Sequence variants lacking this *Tsp509I* site were detectable at passage 7 and had completely replaced the parental sequence by the 13th passage in infected cells (Fig. 3A, lane 2). Restriction enzyme polymorphisms corresponding to two other amino changes (312 Ile → Met and 594 Phe → Ser) were not detected in the bulk RT-PCR products from infected cells, implying that these changes were present in only a small fraction of the passaged vector or that they were PCR artifacts captured during cloning.

To determine whether the variant sequences conferred a growth advantage, we electroporated BHK cells with a 10:1 mixture of RNA from the original Sindbis mCAT1 clone versus one of the clones lacking the *Tsp509I* site (clone 1). In one experiment (Fig. 3A, lanes 4–7), the *Tsp509I* variant increased from low levels at early passages to ~40% by passage 6. This pattern of moderate increase in the proportion of sequences lacking the *Tsp509I* site was seen in a second competition experiment, whereas in two other experiments the *Tsp509I* variant was lost during serial passage. Loss of the *Tsp509I*-negative variant in some experiments does not rule out selective advantage, because vectors retaining the *Tsp509I* site could have accrued other mutations conferring selective advantage (see later).

Another way to evaluate the significance of the phenylalanine-to-isoleucine change is to repeat the serial passage and see whether the same change occurs. A second serial passage was performed starting with the wild-type pSINmCAT1 vector. At various passage levels, RNA was isolated from cells, and a segment of mCAT1

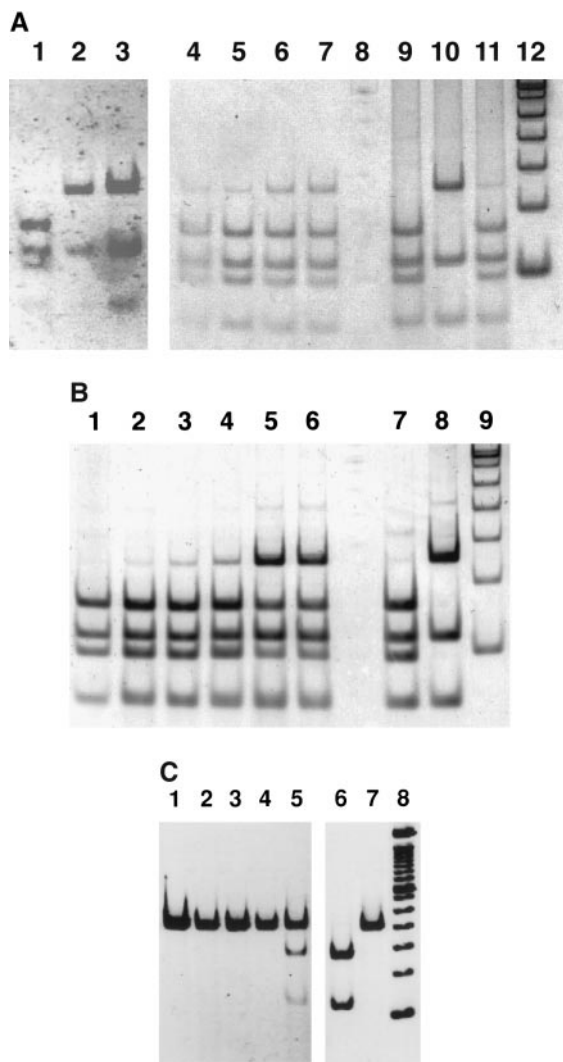


FIG. 3. Evolution of pSINmCAT1 RNA *in vivo*. Restriction fragment polymorphism analysis of a 430-bp segment of the mCAT1 gene containing the third extracellular loop. DNA was obtained by RT-PCR from cloned plasmids or RNA from cells infected with serially passaged pSINmCAT1. PCR products were digested with *Tsp501I* (A and B) or *BstUI* (C), electrophoresed in acrylamide, and stained with ethidium bromide. Panels A and B show the evolution of variant sequences lacking a *Tsp501I* site, which results in a 257-bp fragment, rather than 95- and 162-bp fragments. (A, lanes 1–3) Serial passage beginning with wild-type vector; cellular RNAs for RT-PCR were from the 7th, 13th, and 20th passages, respectively. Lanes 4–7, a competition serial passage experiment beginning with a mixture of 90% wild-type–10% clone 1 vector RNA. RNAs were from the 3rd, 4th, 6th, and 7th passages, respectively. Lane 8, (dilute) 100-bp ladder. Lanes 9–11, PCRs were from DNA from wild-type vector, clone 1, or a 9:1 mixture of the two, respectively. Lane 12, 100-bp ladder marker. (B) Repeat serial passage experiment beginning with wild-type vector. Lanes 1–6, RT-PCR began with cellular RNA from passages 1, 4, 7, 9, 13, and 14, respectively. Lanes 7 and 8, PCR began with DNA from wild-type plasmid and clone 1, respectively. Lane 9, 100-bp ladder marker. (C) Evolution of variant sequences gaining a *BstUI* site from the same serial passage experiment shown in panel B. Lanes 1–5, RT-PCR began with cellular RNA from passages 1, 4, 7, 9, and 13, respectively. Lanes 6 and 7, PCR began with DNA from clone containing *BstUI* site or wild-type vector, respectively. Lane 8, 100-bp ladder marker.

containing the third extracellular loop was amplified by RT-PCR and analyzed by *Tsp509I* digestion. As shown in Fig. 3B, the *Tsp509I* site was again lost in an increasing proportion of passaged vector. RT-PCR products from the 12th passage were cloned, and three clones lacking the *Tsp509I* site as well as six clones containing the *Tsp509I* site were sequenced. All three clones lacking the *Tsp509I* site were found to have a T-to-C change at base 670 corresponding to a phenylalanine-to-leucine substitution at amino acid 224. Thus the phenylalanine was replaced by leucine instead of isoleucine in the second experiment. In addition, one of the clones had a C-to-T change at position 761 corresponding to a threonine-to-isoleucine substitution at amino acid 254. Interestingly, all of the clones containing the wild-type *Tsp509I* sequence had a T-to-C change at position 698 corresponding to a valine-to-alanine substitution at amino acid 233. This T-to-C change at position 698 led to the gain of a *Bst*UI site. *Bst*UI digestion of RT-PCR products from total cell RNA at different passage levels showed that the *Bst*UI variant constituted ~40% of the vector RNA at passage 13 (Fig. 3C). Thus a large proportion of the sequence variants that retained the phenylalanine at position 224 were not actually "wild type" but had undergone selection for another amino acid substitution in the third extracellular loop of mCAT1.

## DISCUSSION

Our data show that alphavirus vectors encoding the mCAT1 receptor can spread in tissue culture in cells expressing a fusogenic form of MLV envelope. Thus the phenomenon of "minimal viruses" derived from alphavirus vectors encoding fusogenic proteins (Rolls *et al.*, 1994) can be extended to vectors encoding a viral receptor instead of viral envelope. Although previous reports showed that vectors encoding VSV-G protein and a truncated, fusogenic form of MLV envelope lead to infectious vesicles and spreading infection (Rolls *et al.*, 1994; Lebedeva *et al.*, 1997), it is important to point out that not all viral envelopes and receptors lead to infectious particles in this system. Semliki vectors encoding rabies virus envelope spread poorly (Rolls *et al.*, 1994), and we have so far been unsuccessful in generating spreading infection with vectors encoding HIV env or CD4 plus CXCR4, despite their ability to induce fusion in cells expressing the cognate fusion proteins. What features are required for spreading infection beyond cell surface expression and the ability to induce fusion are, so far, unknown.

The appearance of infectious particles in the supernatant correlated roughly with the onset and extent of cytopathic effect in infected BHK cells, suggesting that infectious particle formation may be a relatively nonspecific effect of cell degeneration. A low rate of cell degeneration with release of vesicles has been reported in normal cell lines and is accelerated by apoptosis (Zhang

*et al.*, 1998). Sindbis vectors shut off host cell synthesis and cause cytopathicity after 30–40 h (Frolov and Schlesinger, 1994), which may be related to apoptosis seen with full-length Sindbis virus (Nava *et al.*, 1998; Karpf and Brown, 1998; Joe *et al.*, 1998). Sindbis RNA replicates in association with cell membranes (Froshauer *et al.*, 1988), so cell fragmentation associated with necrosis or apoptosis could release vesicles carrying vector RNA. On the other hand, the production of infectious vesicles cannot be solely a consequence of Sindbis vector replication, because not all vector-encoded fusion proteins lead to infectious vesicles. The ability of some vector-encoded fusion proteins to generate infectious particles could be related to their interaction with cellular proteins or lipids normally involved in vesicle formation. The mCAT1 protein colocalizes in the plasma membrane with caveolin (McDonald *et al.*, 1997), a protein involved in vesicle formation and associated with specialized lipid domains known as "rafts" (Li *et al.*, 1998; Reitveld and Simons, 1998).

We initially hoped that the alphavirus-mCAT1 vector would provide a model for vectors that specifically infect (and kill) retrovirus-infected cells. In the case of MLV, this was not possible because chronically infected cells express the immature, nonfusogenic form of envelope on their surface. The fact that cells bearing the immature envelope were not susceptible to infection provides strong evidence that infection in this system involves fusion rather than just binding, which is mediated by the SU portion of the MLV envelope. This contrasts with recent studies in which Sindbis vectors were targeted to various cells by inserting a segment of protein A into the Sindbis E2 envelope gene and mixing mutant virus particles with IgG specific for target cell surface antigens (Ohno *et al.*, 1997). Curiously, the Sindbis E1 protein, which mediates virus-cell fusion, was not detected in these particles, raising the possibility that these vectors entered cells via an alternative mechanism.

Passage of a gene like mCAT1 in alphavirus vectors with continued selection for fusogenicity may be a good way to search for functional variants in a receptor. The rate of mutation of the CAT1 gene when inserted into the alphavirus vector is orders of magnitude higher than when inherited as a normal mouse gene. We found eight amino acid changes in full-length clones of amplified receptor after 12 serial passages and three more in clones of a 430-bp segment containing the third extracellular loop of mCAT1. Because we analyzed cloned inserts amplified by RT-PCR, the possibility of RT or PCR artifacts is of obvious concern. The fact that none of the changes in the full-length clones abolished functionality provides circumstantial evidence that the changes were not artifacts. Also, the number of mutations we observed is higher than expected for RT-PCR but consistent with mutation rates of RNA polymerases: 16 nucleotide changes (counting the 9-base deletion as one change) in

6 kb of sequenced DNA corresponds to an error rate of  $2.7 \times 10^{-3}$ , which is  $\sim 7$  times higher than expected for RT ( $\sim 10^{-4}$ ) followed by 30 cycles of PCR with an error rate of  $10^{-5}$ /cycle. The observed rate of  $2.7 \times 10^{-3}$  after 12 serial passages is consistent with the mutation rate of many RNA polymerases ( $\sim 10^{-4}$ ) and the observation that many RNA viruses propagate as a swarm of RNA quaspecies (Eigen, 1993; Domingo and Holland, 1997).

Three of the mutations, the phenylalanine-to-leucine and -isoleucine changes at amino acid 224 and the valine-to-alanine change at amino acid 233, could not have been artifacts because restriction analysis of bulk RT-PCR products showed that vectors with these sequences constituted a significant fraction of total vector RNA at several times during passage (Fig. 3). The location of these changes fits with observations that residues at nearby sites in the third extracellular loop of CAT1 affect envelope binding and are responsible for differences in receptor function between homologues of mCAT1 (Albritton *et al.*, 1993; Kavanaugh *et al.*, 1994; Kim and Cunningham, 1993; Yoshimoto *et al.*, 1993; Eiden *et al.*, 1993, 1994). The change from phenylalanine at position 224 to leucine or isoleucine in independent experiments argues strongly that changes at position 224 are selected.

The reason for the selective advantage of some mCAT1 sequence variants in this system is not yet clear. They could lead to increased fusion between membranes containing envelope and receptor, or they could affect processes specific to the Sindbis vector system such as formation of vector RNA-containing vesicles or details of cell-to-cell spread. We did not detect a difference in the titer of infectious vesicles or plaque size when we compared vectors encoding wild-type versus variant mCAT1 sequences, but these assays are not very precise. We also failed to detect a difference in the titer of MLV particles assayed on BHK cells chronically expressing the wild type versus a variant mCAT1 gene. However, a small increase in the rate of spread of a vector encoding a variant mCAT1 gene could lead to a large increase in the relative amount of that vector after multiple cycles because of the exponential nature of growth. For alphavirus vectors, the cycle time could be as short as 8 h, so 12 serial passages with growth for 24–48 h during each passage could represent more than 30 cycles.

This system may provide a useful method for generating diversity in viral receptor genes while maintaining selection for fusogenicity. The low titer of infectious vesicles in this system would appear to pose a limitation, because it suggests that only a small number of mutations would be screened in any experiment. However, most spread of vector in this system probably occurs through cell–cell contact rather than the formation of infectious particles in the supernatant, and in this setting, it is difficult to estimate the number of variant sequences

generated and the number of such sequences exposed to selection via expressed proteins. Empirically, finding similar mutations in 1 particular amino acid of 622 amino acids in mCAT1 in independent experiments suggests that most single base mutations are probably sampled in each experiment and, remarkably, that the number of strongly selected mutations is small. Two related systems have been reported in which receptor genes were substituted for virus envelope genes in rapidly replicating vectors. In one, mCAT1 and an avian leukosis virus receptor were inserted into a retroviral vector and shown to produce virus particles that infected cognate envelope-expressing cells (Baillet and Bates, 1998). In the other, rhabdovirus vectors encoding HIV receptors were used to target HIV envelope-expressing cells (Schnell *et al.*, 1997; Mebatsion *et al.*, 1997). Apart from their interest as potential “magic bullets” to target virus-infected cells, these systems could be useful for identifying receptor variants with potentially useful properties such as altered receptor binding.

## MATERIALS AND METHODS

### Vectors

The vectors used in this study are shown schematically in Fig. 1. pSINmCAT1 was made by transferring a 2.2-kb *Bam*HI–*Stu*I fragment containing the CAT1 gene from pJET (Albritton *et al.*, 1989) to a *Bam*HI–*Sma*I-digested pGEM7Z plasmid (Promega, Madison, WI). The mCAT1 gene was cut out of this plasmid as an *Mlu*I–*Bsp*120I fragment and transferred to pSinRep5 (Invitrogen, Carlsbad, CA) cut with the same enzymes (Fig. 1, first line).

pSFVlacZ-MLVenv12 is a double subgenomic promoter Semliki Forest virus vector containing *lacZ* after the 5′ subgenomic promoter and MLV envelope with a stop codon at the normal p12e cleavage site after the 3′ subgenomic promoter. This vector was made by taking a *Bgl*II–*Sma*I fragment containing the subgenomic promoter and *lacZ* from pSFVlacZ (Life Technologies, Gaithersburg, MD), converting the *Sma*I site to *Bgl*II with a linker, and inserting this fragment into pSFVMenv-tr (Lebedeva *et al.*, 1997) cut with *Bgl*II (Fig. 1, second line).

pMLVenv12 is a stable expression vector for MLVenv12e that was derived from pABWN (Niwa *et al.*, 1991), a plasmid containing a TK promoter driving the neomycin resistance gene, followed by a hybrid CMV-IE-enhancer/chicken actin promoter upstream of a multicloning site. We began with a derivative of pABWN, pCD4Fed (Matano *et al.*, 1995), that was kindly provided by Tetsuro Matano (AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan). pCD4Fed contains, in the multicloning site of pABWN, the human CD4 gene fused to F-MLVenv with a stop codon at the normal p12e cleavage site. We digested pCD4Fed with *Xba*I and *Cla*I to remove CD4 and F-MLV env sequences

up to a *Cla*I site just upstream of the p12e stop codon. An *Xba*I-*Cla*I fragment containing M-MLV env sequence from the initiation codon to the homologous *Cla*I site was then inserted. The resulting expression vector for fusogenic MLVenv is designated pMLVenv12 (Fig. 1, third line).

## Cells

BHK cells (American Type Culture Collection, Gaithersburg, MD) and NIH3T3 cells (Janet Hartley, National Institutes of Health, Bethesda, MD) were grown in DMEM (Life Technologies) supplemented with 10% FCS. NIH3T3 cells chronically infected with M-MLV are designated M-MLV-NIH3T3. BHK cells stably expressing MLVenv with a stop codon at the normal p12e cleavage site were obtained through transfection with plasmid pMLVenv12 and selection with 0.4–0.8 mg/ml G418 (Life Technologies). Colonies were screened for expression of MLVenv by flow cytometry using goat anti-Rauscher MLV env serum (National Cancer Institute, Bethesda, MD). One such cell line is designated BHK-MLVenv12.

## Vector RNA and electroporation

Approximately 1  $\mu$ g of *Pac*I-linearized Sindbis plasmid DNA was transcribed *in vitro* using SP6 RNA polymerase (Promega, Madison WI) and 7-methyl-G (New England BioLabs, Beverly, MA). The transcription mixture was added to  $10^{6-7}$  BHK cells in 0.4 ml of PBS and pulsed twice using a Bio-Rad (Hercules, CA) electroporation device set to 0.8–1.2 kV and 25  $\mu$ F.

## Infection and plaque assay

Cell lysates were made by suspending cells harvested with a rubber policeman in 1 ml of DMEM and breaking them open with a Dounce homogenizer (50 strokes) or by vigorous shaking with 0.5-mm glass beads in a MiniBeadbeater (Biospec Products, Bartlesville, OK) or through ultrasonication for 30 s in a Torbeo Ultrasonic Cell Disruptor (Cole-Parmer Instrument Co., Vernon Hills, IL). Cell lysates or supernatants were passed through 0.45- $\mu$ m filters. BHK-MLVenv12 cells were infected when ~70% confluent by incubation with 0.5–1 ml of filtered cell lysate or supernatant in DMEM–1% FCS supplemented with 8  $\mu$ g/ml Polybrene. After 4 h, the inoculum was replaced with DMEM–1% FCS, and the cells were fixed and stained 24–48 h later with methanol containing 0.17% (wt/vol) carbol Fuchsin and 0.5% methylene blue. Syncytia were counted under a dissecting microscope.

## RT-PCR and assays for *Tsp*509I and *Bst*UI restriction site polymorphisms in mCAT1

RNA from infected cells was prepared using Trizol (Life Technologies) and amplified with a Titan RT-PCR kit (Boehringer Mannheim, Indianapolis, IN). For RT-PCR of

the full-length mCAT1 gene in Sindbis vector RNA, we used reverse primer 5'-ACCAGCCTGATGCATTATGC and forward primer 5'-CTACGGTGGTCCTAAATAGTCAGC, which correspond to sequences in the 3' untranslated region and subgenomic promoter flanking mCAT1 in pSINRep5. To amplify a 430-bp segment containing the third extracellular loop of mCAT1 (bases 399–828), we used reverse primer 5'-CTGGGGGTTCTTGACTTCTTCC and forward primer 5'-TGCGACTTTTGACGAGCTGATAG. This PCR product was digested with *Tsp*509I, which cuts at positions 513, 573, and 667 in the wild-type mCAT1 sequence, or with *Bst*UI, and the digestion products were electrophoresed in 6% acrylamide gels.

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